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Absence of HHV-6 and HHV-7 in cerebrospinal fluid in relapsing–remitting multiple sclerosis

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Objective – To contribute to clarifying the controversy on the association between Human Herpesviruses 6 and 7 (HHV-6, HHV-7) and multiple sclerosis (MS) studying patients with relapsing–remitting MS (RRMS) with or without evidence of disease activity (clinically or radiologically evaluated). *Material and methods* – In 25 RRMS patients, 7 suspected MS patients and 9 patients with other neurological diseases, the following parameters were analysed: i) antibody titers (IgM and IgG) against HHV-6 by indirect immunofluorescence both in serum and cerebrospinal fluid (CSF) samples; ii) PCR-detection of HHV-6 DNA and HHV-7 DNA in CSF and HHV-6 DNA in peripheral blood mononuclear cells (PBMCs). MS patients in remission underwent a gadolinium-enhanced magnetic resonance imaging in proximity of sample collections. *Results* – No viral DNA was found in any CSF sample, HHV-6 DNA frequency in PBMCs of MS patients and controls was not statistically different. Antibody titers against HHV-6 were comparable to those of the general population. Some 30.4% of MS patients were seronegative to HHV6. *Conclusion* – Our data suggest that there is no relationship between HHV-6 or HHV-7 and MS.

Key words: herpesvirus; HHV-6; HHV-7; relapsing–remitting multiple sclerosis

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The central nervous system (CNS) has been considered a site of latency of Human Herpes Virus 6 (HHV-6) (1–5).

The HHV-6 may cause disease defined as exanthem subitum in infancy but often the infection is asymptomatic. HHV-7, another β-herpesvirus, is also implicated in similar disease. Neuroinvasion by HHV-7 has been suggested by some cases of encephalopathy associated with febrile exanthem due to HHV-7 infection (6, 7).

Viral infections in general have been proposed as triggering factors for multiple sclerosis (MS) (8, 9) and in this context major suspects should be viruses capable of persistent infections such as herpesviruses. In addition a typical property of herpesviruses is latent infection with possible reactivation which could play a role in a relapsing–remitting pattern of disease.

Whether HHV-6 has a role in the pathophysiology of MS has yet to be determined. Several studies suggested an association between HHV-6 and MS showing: i) higher levels of anti HHV-6 antibodies in MS patients than in controls in serum or cerebrospinal fluid (CSF) (10–13); ii) HHV-6 DNA detection in serum, peripheral blood mononuclear cells or CSF (12–16) in some MS patients but never in controls, iii) association between HHV-6 infection of CNS and demyelinating lesions in histopathologic studies (17–21); iv) the expression of HHV-6 antigens in the nuclei of oligodendrocytes (or small cells whose appearance was consistent with oligodendroglial cells) most commonly within plaques in brain specimens from MS patients but not in controls (3, 22, 23). By contrast, the latter intriguing observations were not confirmed by others (24) and other studies did not demonstrate

igher levels of anti HHV-6 antibodies (25, 26) or igher prevalence of HHV-6 DNA in peripheral lood mononuclear cells, serum or CSF (10, 25, 27–0) in MS patients as compared to controls.

Published data on HHV-7 in MS are few but all onsistent with the absence of HHV-7 in the CSF nd serum of MS patients (14, 30).

However, limited data are available on herpes-iral status in CSF in MS patients during evidence f disease activity.

For this reason we studied PCR-detection of HHV-6 or HHV-7 DNA in CSF and HHV-6 DNA n peripheral blood mononuclear cells (PBMCs) in atients with: i) relapsing–remitting MS (RRMS), vith or without evidence of disease activity; ii) uspected MS; iii) other different neurological liseases. Disease activity at the time of sampling vas well documented either clinically or through the resence of gadolinium-enhanced lesions on brain MRI. Furthermore, to compare the results con-erning DNA detection in CSF to the presence of an ntibody response to HHV-6, the titers of Ab anti-HHV-6 (IgM and IgG) were studied both in serum nd CSF.

Material and methods

Twenty-five patients affected by clinically or aboratory-supported definite or probable RRMS 31) with or without evidence of disease activity vere studied. These were a subset of patients consecutively referring to a neurologic department o have the first diagnosis, a diagnostic confirmation or because of a severe relapse for which hospital admission had been requested. They were all ncluded in a regular follow-up. Seven patients vith suspected demyelinating disease who did not atisfy diagnostic criteria for definite or probable MS and 9 patients diagnosed as having other neurological diseases (including meningioma, migraine, acute or chronic inflammatory demyeli-nating polyradiculopathy, cerebrovascular disease, neurological complications in Sjogren's syndrome; amyotrophic lateral sclerosis) hospitalized in the same department were also studied.

Demographic and clinical features for each subgroup considered are reported in Table 1.

Eleven MS patients were analysed during a relapse defined according to current criteria (32) with onset within 3 weeks from sample collections. Fourteen MS patients were in remission. For the latter a gadolinium-enhanced magnetic resonance imaging (Gd-MRI) of the brain was carried out in proximity of sample collections. Enhancement was detected by a delayed T1-weighted scan after an i.v. bolus injection of a single dose of gadolinium-DTPA.

All MS subjects were free of symptoms of infection or coincidental inflammatory conditions or other immunologic mediated disorders except for one with celiac disease and psoriasis.

To evaluate the presence of concomitant infectious or inflammatory conditions routine blood and urine samples were collected.

Fourteen MS patients were under steroids (generally, high dose methylprednisolone i.v. 1 g/die for 5 days and the patients were in treatment or in suspension within almost 3 weeks) and 11 were not. This treatment could possibly play a role in herpesviral reactivation. No other immunosuppressive drugs were used.

Consent was obtained from all participating subjects and the study was carried out according to the principles of the Helsinki Declaration.

Blood and CSF samples were obtained on the same day in order to analyse: i) antibody titers (IgM and IgG) against HHV-6 by indirect immunofluorescence both in serum and CSF samples; ii) PCR detection of HHV-6 DNA and HHV-7 DNA in CSF; iii) PCR detection of HHV-6 DNA in PBMCs. Mean time interval between sample collections and presumed onset of a relapse was 12.9 days (SD = ±6.4; median = 13; range = 1–21). The mean time interval between sample collections and the Gd-MRI was 4.2 days (SD = ±3.5; median = 4; range: 1–10).

Except for one case (in which the CSF was obtained during neurosurgical intervention), all CSF samples were obtained from routine lumbar punctures.

Table 1. Demographic and clinical features of subgroups

Subgroups	Gender (M/F)	Age (years) Mean ± SD	Median	Range	Disease duration (months) Mean ± SD	Median	Range	EDSS* Mean ± SD	Median	Range	Steroids (patients)
MS in relapse ($n=11$)	1/11	38.5 ± 10.1	37	25–53	28.3 ± 56.1	1	1–172	3.3 ± 2.2 (0.4 ± 0.6)	2.5 (0)	0–9 (0–2)	9
MS in remission Gd − ($n=9$)	5/4	36.8 ± 11.5	37	21–49	72.3 ± 76.9	41	3–237	1.8 ± 1.2	2	0–3.5	3
MS in remission Gd + ($n=5$)	2/3	34.4 ± 10.4	37	24–47	78.8 ± 75.5	90	2–175	0.8 ± 0.7	1	0–1.5	2
Suspected MS ($n=7$)	2/5	38.3 ± 9.6	40	21–50	/	/	/	/	/	/	2
Other ($n=9$)	3/6	47 ± 18.9	43	23–79	/	/	/	/	/	/	2

* EDSS features at the moment of serum/CSF sampling; pre-relapse EDSS features are reported in brackets.

DNA extraction and amplification

One ml of CSF from each subject was centrifuged in a 1.5 ml microcentrifuge tube for 20 min at $21,000 \times g$ in order to pellet cells and herpesviral particles. The pellet was frozen at −20°C for DNA extraction.

Subsequently 10^6 PBMCs were separated from EDTA treated blood after a standard Fycoll density gradient centrifugation.

DNA was extracted by the following procedure in a 40 ml reaction containing: 10 mM TRIS-HCL ph 8, 100 mg/ml proteinase K, 0.5% Nonidet P40 and 0.5% Tween 20 for 1 h at 50°C. Five ml of this reaction were added to 85 ml of the amplification reaction buffer (1.5 mM MgCl) and incubated at 94°C for 7 min to inactivate proteinase K. After the addition of nucleotides, primers and a thermostable DNA polymerase (DyNAZyme DNA Polymerase, Finnzymes Oy, Espoo, Finland) the amplification reactions were carried out for 50 cycles. In order to guarantee maximum sensitivity the primers were carefully selected so that they could efficiently amplify up to single HHV-6 DNA molecules after 50 amplification cycles (verified by a Poisson distribution of positives at end point dilution). For HHV-6 the following two primer pairs were chosen (5'-3' as synthesized): 1) GAATATCTT-CTTTTACATCC sense and TGTTTCATGGCA-GCCTTCA antisense, spanning 85 base pairs in the immediate early genes region; 2) TAAACATCA-ATGCGTTGCATACAG sense and GTGTTT-CCATTGTACTGAAACCGGT antisense, spanning 783 base pairs in the major capsid protein region. Both primer pairs were capable of detecting subtype A as well as subtype B of HHV-6.

For HHV-7 the following primer pair was used: TAGTTCCAGCACTGCAATCG sense and CAC-AAAAGCGTCGCTATCAA antisense, spanning 408 base pairs in the major capsid protein region.

Antibody detection

Antibody titers against HHV-6 were determined by use of a commercial indirect immunofluorescence test (HHV-6 IFA, ALIFAX, Padova, Italy), following the manufacturer's instructions. Titer of 1/32 and 1/8 were established as thresholds for positivity respectively in serum and CSF.

Results

No HHV-6 or HHV-7 DNA was found in any CSF sample. PBMCs from 14 MS patients (2 in relapse) and 9 controls were analysed for HHV-6 DNA. Two patients (in remission) out of 14 were found positive by PCR and none of the controls, this difference being not statistically significant.

Antibody titers were analysed in CSF of 25 MS patients. All subjects displayed $<1:8$ IgG and IgM titers in CSF (with the exception of 1 MS patient in remission who displayed a 1:8 IgG titer in CSF). In serum antibody titers were determined in 23 MS patients and 8 controls (serum was not available for 2 patients in relapse and 1 control with meningioma). All subjects analysed displayed $<1:32$ IgM titers in serum. Seven out of 23 (30.4%) MS patients displayed a $<1:32$ IgG titer against HHV-6. This result is comparable to that of the general population from the same geographical area (39% in 97 age-matched subjects with irrelevant disease analysed in the same lab). The percentage of seronegative subjects was higher (75%) in the other neurologic disease subgroup used as control although this difference was not statistically significant ($\chi^2 = 3.2$, d.f. $= 1$, $P = 0.07$). In addition, the titer distribution among seropositive patients (although in limited numbers) did not suggest a significant difference with the general population: 1:32 7/23 (30% vs 29%), 1:64 5/23 (22% vs 12%), 1:128 or higher 4/23 (18% vs 20%).

Discussion

Serologic studies in MS are difficult to interpret due to dysfunction in immunoregulation and/or the genetic background of MS patients which could explain increased antibody titers in MS, as also discussed by Fillet et al. (27). Moreover, even if the presence of HHV-6 in plasma could be of interest since in normal individuals HHV-6 DNA was not demonstrated (33), it cannot be interpreted as direct evidence of reactivation of HHV-6 in the central nervous system. This is the reason why our study focused on the detection of β-herpesviruses in CSF.

However, three major issues must be considered also in a CSF study: i) investigations should be carried out on patients with early stages of the disease as suggested by Fillet et al. (27); ii) the expression of HHV-6 could be linked to the active phase of the disease, but the time gap between reactivation of the virus and sampling of CSF could be critical to detect such a link; iii) plaque-associated expression of HHV-6 could not be detected by PCR performed on CSF samples.

As far as the first issue is concerned, the majority of MS patients included in the present study were at an early stage of the disease (median of duration = 11 months) with low EDSS (all ≤ 3.5 out of relapse).

As far as the second and third issues are concerned, the present research was aimed at

tudying patients both with or without evidence of lisease activity (clinically or radiologically assessed) ınd CSF samples were obtained within 3 weeks at nost (median = 13 days) from presumed onset of a elapse. However, to our knowledge no data are ıvailable about the duration of β-herpesvirus DNA letection in the CSF after reactivation (if it ever ɔccurs) in the CNS. Although we cannot exclude hat the method of detection of β-herpesvirus DNA ve used could have missed a faint β-herpesvirus ıctivity in CSF, no other technique can be definitely ɔonsidered more sensitive than PCR. On the other ıand, 5 MS patients with positive serum IgG (i.e. exposed to HHV-6 in their life), who were analysed within 21 days at most from the evidence of clinical relapse, had neither HHV-6 IgM in the serum nor IgG or IgM in the CSF. The same findings were observed in MS patients in a remitting stage but with enhancing lesions on the MRI scan. Although the commercial antibody detection assay used in this study might not be extremely sensitive, it is unlikely that a reactivation of HHV-6 with abundant production of viral proteins (and possibly viral particles) as previous results suggest (3), occurred in our MS patients. In addition a proportion of MS patients resulted seronegative against HHV-6 suggesting that they never came in contact with the virus or that they overcame the infection very efficiently several years before, forcing the virus into a very deep and irreversible latency with very low residual antibody production. The same conclusion seems true according to the results concerning HHV-6 DNA in PBMCs, which show that no evident systemic reactivation of HHV-6 occurs in MS patients, confirming some previous observations (5, 10, 15).

Our results seem to exclude the possible pathogenetic mechanism hypothesized of a direct cytopathic effect on olygodendroglia by HHV-6 or HHV-7 in MS in agreement with some of the previous reports quoted in the introduction. However, the positive correlation between HHV-6 infection and MS suggested by some authors must be taken into consideration (3, 11–13, 16, 22). The possibility is still open that in a small subset of MS patients reactivation of HHV-6 occurs and might have a pathophysiological role. In other words, heterogeneity in the pathophysiology of MS could include a potential role for HHV-6 in triggering the dysimmune process, through direct or indirect effects, in predisposed hosts who are a minority of the MS population. Such a role might be in terms of activation of the immune system, as hypothesized, in more general terms, for other viruses or intracellular agents (8, 34, 35). Another possible pathogenetic mechanism is the activation of other viruses – e.g. the MS associated retrovirus described by Perron et al. (36). On the other hand, since the ability of HHV-6 to survive in infected cells necessarily depends on its capacity to evade immune system control, it is possible that HHV-6 expression described in some MS cases could be just an epiphenomenon due to the particular immune or genetic background of those patients without any real etiopathogenetic role.

In conclusion our results do not confirm previous data that suggested the role of the reactivation of HHV-6 in CNS as a triggering factor for the demyelinating process in MS.

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